Studies on the "Messenger" Activity of RNA Synthesized with RNA Polymerase

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INTRODUCTION

The messenger hypothesis, which was discussed extensively at this symposium two years ago (1961), is now supported by several lines of direct and circumstantial evidence (for recent review see Simpson, 1962). The hypothesis provides a specific model for the genetic control of protein synthesis, the central feature of which is the transcription of DNA nucleotide sequence into complementary sequences in the messenger RNA (Jacob and Monod, 1961a; 1961b).

The DNA-dependent RNA polymerases, whose mode of action and wide distribution in nature are now well documented (Geiduschek, Nakamoto and Weiss, 1961; Stevens, 1961; Burma et al., 1961; Furth, Hurwitz, and Anders, 1962; Chamberlin and Berg, 1962), provide a plausible enzymatic mechanism for the production of messenger RNA. Although the present evidence is largely indirect, these enzymes are therefore assumed to play an integral role in protein synthesis.

More direct evidence should be obtainable through study of reconstructed in vitro systems. If cell-free amino acid incorporation reflects the mechanism of specific protein synthesis in the intact cell, then the messenger model makes two predictions which can be experimentally tested:

(1) RNA synthesized in the presence of the polymerase and a DNA template should stimulate amino acid incorporation.

(2) If, in the cell-free system, specific proteins are synthesized de novo under the influence of this RNA, their nature should be determined by the DNA used to direct RNA synthesis.

Our studies until now have been concerned with examining the first prediction (Wood and Berg, 1962). The results show that RNA synthesized enzymatically in the presence of a native DNA template is active in stimulating amino acid incorporation. Denatured or single-stranded DNA, however, appears to be incapable of directing the synthesis of active RNA. This unexpected finding is further explored in the present study.

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NATURE OF THE SYSTEM AND EFFECTS OF T2 RNA ADDITION

The experiments to be reported were carried out using a soluble protein-ribosome system (SPR system) from *Escherichia coli*. This consisted basically of washed ribosomes and a soluble protein fraction from which endogenous nucleic acid and some inactive protein had been removed by protamine sulfate precipitation. These components were supplemented with the soluble cofactors required for maximal activity (see legend to Table 1). Details of the system and the method of assay have been previously reported (Wood and Berg, 1962).

As noted by several investigators, the amino acid incorporation reaction in such systems is of short duration and quite limited extent (Lamborg and Zamecnik, 1960; Tissières, Schlessinger and Gros, 1960; Wood and Berg, 1962). The final level of incorporation is proportional to the amount of ribosomes added (Wood and Berg, 1962), suggesting that some ribosomal component is limiting. Since it seemed possible that this might be endogenous messenger RNA which was destroyed during the reaction, RNA synthesized with RNA polymerase was tested for its ability to supplement the limiting ribosomal component and increase the extent of amino acid incorporation.

In the initial experiments, purified RNA polymerase (Chamberlin and Berg, 1962), T2 DNA, and the necessary ribonucleoside triphosphates were added directly to the SPR system. Addition of these components together increases the extent of amino acid incorporation up to 20-fold (Table 1). The increased incorporation is entirely dependent on the presence of both the soluble and ribosomal fractions and still requires all of the soluble cofactors mentioned above (Wood and Berg, 1962); therefore, it presumably represents a stimulation of the original low activity rather than another pathway of amino acid incorporation such as that reported by Beljanski (1960) or Kaji, Kaji and Novelli (1963). The presence of DNase in the complete reaction mixture or the omission of either the T2 DNA or the polymerase largely eliminates the increased activity, suggesting that stimulation depends upon RNA

TABLE 1. STIMULATION OF AMINO ACID INCORPORATION IN THE SPR SYSTEM BY RNA POLYMERASE AND T2 DNA

Additions to SPR system	Leucine-Valine incorporation
	cpm
None	150
RNA polymerase and T2 DNA	3080
RNA polymerase	210
T2 DNA	420
RNA polymerase and T2 DNA plus DNase	520
RNA polymerase and T2 DNA minus ribosomes	80
RNA polymerase and T2 DNA minus soluble fraction	10

Data are taken from Wood and Berg (1962). Incubation was for 60 min at 37° in a final volume of 0.25 ml. The SPR system consisted of ribosomal (250 μg) and soluble (400 μg) protein fractions described in the text, Tris buffer, pH 7.8, and the soluble cofactors magnesium acetate, KCl, β-mercaptoethanol, ATP, GTP, UTP, CTP, *E. coli* amino acid-acceptor RNA, and a mixture of amino acids, including C^{14}-DL-leucine (1.5×10^7 cpm/μmole) and C^{14}-DL-valine (1.4×10^7 cpm/μmole). Additions where indicated were purified RNA polymerase (30 μg), T2 DNA (60 mμmoles of nucleotide), and crystalline bovine pancreatic DNase (1.25 μg). Incorporation was measured as conversion of the C^{14}-labeled amino acids into a hot perchloric acid-insoluble form.

synthesis. This conclusion is supported by the finding that addition of isolated enzymatically synthesized T2 RNA to the SPR system, in the absence of the enzyme and DNA, also stimulates amino acid incorporation, although somewhat less strikingly (Fig. 1). (Possible reasons for the smaller stimulation have been discussed previously [Wood and Berg, 1962; Ning and Stevens, 1962].) This increase is insensitive to DNase, and is proportional to the amount of RNA added up to a saturation level corresponding to approximately one RNA chain of molecular weight 10^5 (Chamberlin, Baldwin and Berg, 1963) per 70S ribosome. Experiments similar to those above have been reported by Ning and Stevens (1962) and Furth, Kahan and Hurwitz (1962).

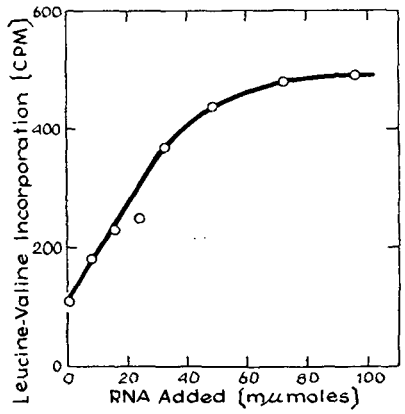

FIGURE 1. Stimulation of amino acid incorporation in the SPR system by isolated T2 RNA. Synthesis of T2 RNA to 2.5-fold excess over the DNA primer was carried out in the presence of RNA polymerase and the four triphosphates. RNA was isolated by phenol extraction of the reaction mixture, passage through a column of Sephadex G-50 over a layer of Amberlite XE-64, and ethanol precipitation, followed by brief dialysis against dilute buffer. The final preparation added to the SPR system (Table 1) contained approximately 20% DNA.

ACTIVITY OF OTHER DNA PREPARATIONS

The foregoing results demonstrate that the first prediction mentioned above is borne out when native T2 DNA is used to direct RNA synthesis. When other DNA preparations are assayed in the SPR system with RNA polymerase (coupled SPR system), the stimulation of amino acid incorporation varies over a wide range (Table 2). Three classes of DNA can be distinguished: "active", producing a 15- to 20-fold stimulation; "slightly active", giving a 1.5- to 3-fold effect; and "inactive", giving no detectable stimulation. From the second column of the table, it can be seen that these variations are not due merely to differences in ability to support RNA systhesis, although some correlation between the two activities is observed. At present,

TABLE 2. CORRELATION OF DNA ACTIVITY IN THE COUPLED SPR SYSTEM WITH ABILITY TO DIRECT ENZYMATIC RNA SYNTHESIS

Source of DNA	Activity in coupled SPR system	Relative rate of RNA synthesis
		per cent
T2 phage	Active	100
T4 phage		65
T6 phage		70
B. subtilis		97
E. coli	Slightly Active	75
Wheat germ		80
Pea seedling		75
Salmon sperm		70
Calf thymus		25
T2 phage (denatured)		20
ϕX 174 phage	Inactive	23

The coupled SPR system contained the components listed under Table 1 with 30 μg of RNA polymerase in addition to 30–60 mμmoles of the indicated DNA preparation. Pea seedling DNA was the gift of Drs. R. C. Huang and J. Bonner; other DNA samples were prepared by published procedures. Designations of DNA activity are explained in the text. Assays for RNA synthesis were performed with 60 mμmoles of DNA and 10 μg of enzyme under the conditions of the SPR system (Tris buffer, pH 7.8, magnesium acetate, KCl, β-mercaptoethanol, ATP, GTP, UTP, CTP); these are essentially similar to the assay conditions described by Chamberlin and Berg (1962) except for the absence of Mn^{++} and the presence of KCl. (DNA preparations from coliphages T5 and T7 and the *Bacillus* phage α also produce a 15- to 20-fold stimulation of amino acid incorporation, but have not been compared for rate of directing RNA synthesis.)

TABLE 3. INACTIVATION OF T2 DNA BY HEAT DENATURATION

Additions to coupled SPR system	Leucine-Valine incorporation
	cpm
None	130
T2 DNA, native	2590
T2 DNA, heat-denatured	140

The coupled SPR system contained the components described under Table 1, including RNA polymerase (30 μg). Native T2 DNA (30 mμmoles) and denatured T2 DNA (30 mμmoles, prepared by heating at a concentration of 2 μmoles/ml in 0.05 M NaCl for 10 min at 100°, followed by rapid cooling) were added as indicated.

reasons for the variations are unclear; however, the studies described below suggest an interpretation based on differences in secondary structure as one contributing possibility.

Heat denaturation of T2 DNA results in almost complete loss of the ability to stimulate amino acid incorporation in the coupled SPR system (Table 3). This result seems paradoxical in light of present knowledge about the RNA polymerase reaction in vitro. It is known that single-stranded DNA will support RNA systhesis, and that both strands of a double-helical DNA are copied by the enzyme (Chamberlin and Berg, 1962); therefore, both native and denatured T2 DNA should give rise to complementary RNA copies of both DNA strands and hence to identical populations of RNA molecules. The above observation, however, as well as the complete inactivity of the single-stranded ϕX 174 DNA (Sinsheimer, 1959) in stimulating amino acid incorporation (Table 2), suggests that the RNA directed by single-stranded DNA is inactive.

POSSIBLE EXPLANATIONS FOR THE INACTIVITY OF SINGLE-STRANDED DNA

PRIMARY STRUCTURE DAMAGE TO DNA

It is conceivable that the inability of heat-denatured DNA to direct active RNA synthesis results not from the change in secondary structure, but from depurination, chain scission, or other damage to the DNA primary structure during the heating process. Since such damage should be irreversible, this explanation can be eliminated if activity is regained upon restoration of double-helical secondary structure.

Two methods were used to restore native secondary structure to heat-denatured T2 DNA. In the experiment summarized in Fig. 2, the denatured material was renatured by incubation at elevated temperature in a solution of high ionic strength (Marmur and Doty, 1961). Changes in secondary structure were followed by equilibrium sedimentation in a CsCl density gradient, taking advantage of the difference in buoyant density between denatured and native DNA (Doty et al., 1960). The density gradient patterns obtained with native, denatured, and renatured T2 DNA, and their activities in the coupled SPR system are shown in the figure. Renaturation of the denatured DNA returns the buoyant density almost to that of the native material and restores more than 90% of the ability to stimulate amino acid incorporation.

A second approach was to convert denatured T2 DNA to a double-stranded form by replication with an excess of DNA polymerase and the four deoxyribonucleoside triphosphates. Again, restoration of native secondary structure as judged by the

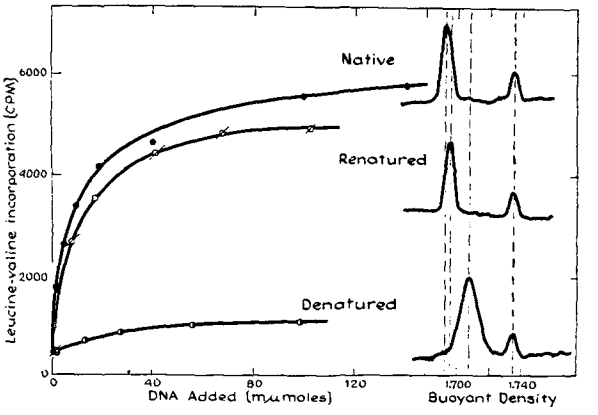

FIGURE 2. Reactivation of heat-denatured T2 DNA by renaturation. T2 DNA was denatured by heating a solution of 60 μg/ml in saline-citrate 0.09 N in Na^+ for 20 min at 100°, followed by rapid cooling. A sample was incubated at 55° in the presence of 0.3 N Na^+ until >90% renaturation had occurred as judged by the hypochromic shift. Native, denatured, and renatured DNA fractions were assayed for activity by adding increasing amounts to the coupled SPR system described in Table 2. For determination of buoyant densities, DNA samples were centrifuged 18 hr at 44,770 rpm in CsCl solutions in a Spinco model E analytical ultracentrifuge equipped with UV optics. A sample of N^{15}-labeled *Ps. aeruginosa* DNA (generously provided by Dr. C. Schildkraut) was included as a reference and assigned a buoyant density of 1.734 g cm^{-3} (see note below). Buoyant densities of the T2 DNA samples, determined from densitometer tracings of UV photographs as described by Schildkraut, Marmur, and Doty (1962), were: native, 1.692 g cm^{-3}; denatured, 1.708 g cm^{-3}; renatured, 1.694 g cm^{-3}.

[**Note:** the buoyant density values reported in this paper are based on a value of 1.670 g cm^{-3} for the density of the synthetic deoxyadenylate-deoxythymidylate (dAT) copolymer (Schachman et al., 1960). This value was obtained in several experiments, using the expression for r_e' derived by Ifft, Voet and Vinograd (1961) to estimate the isoconcentration point in the sector cell. Using dAT copolymer as a reference, the density of the N^{15}-labeled *Ps. aeruginosa* DNA was determined to be 1.734 g cm^{-3}. Assignment of these values results in a scale of densities which is lower than that used by Schildkraut, Marmur and Doty (1962) by a constant increment of 0.008 g cm^{-3}. The difficulty of measuring true buoyant density and the sources of possible error have been discussed by these authors.]

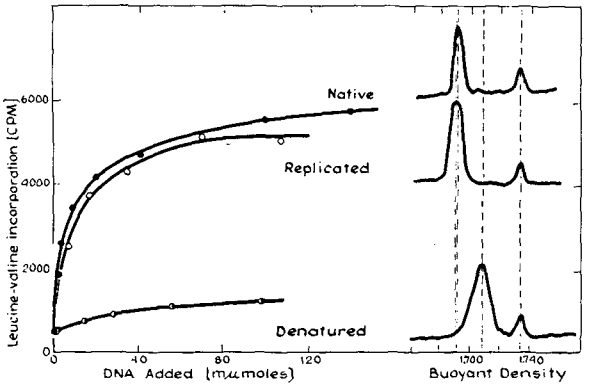

FIGURE 3. Reactivation of heat-denatured T2 DNA by replication with DNA polymerase. A sample of T2 DNA denatured as described under Fig. 2 was incubated with the four deoxyribonucleoside triphosphates and 20 μg of a DEAE fraction of the DNA polymerase (Lehman et al., 1958) until a 1.3-fold replication had occurred as measured by incorporation of dGMP^{32}. The product was deproteinized by passage over Amberlite XE-64 (Chamberlin, Baldwin and Berg, 1963) and dialyzed to remove substrates. Buoyant density determinations and assays of the DNA samples for activity were as described under Fig. 2. The replicated material banded at a density of 1.691 g cm^{-3}.

shift in buoyant density is accompanied by almost complete restoration of activity in the coupled SPR system (Fig. 3). These results rule out irreversible primary structure damage as an explanation for the heat inactivation of T2 DNA; moreover, they strongly suggest a direct correlation between activity and double helical secondary structure.

Further support for this correlation comes from the finding that the inactive single-stranded ϕX DNA is also activated upon conversion to a double-stranded form (Fig. 4). Replication with DNA polymerase as above results in a shift of the buoyant density to a value close to that predicted for double-stranded DNA of this GC content (Schildkraut, Marmur and Doty, 1962). The replicated material produces a significant stimulation of amino

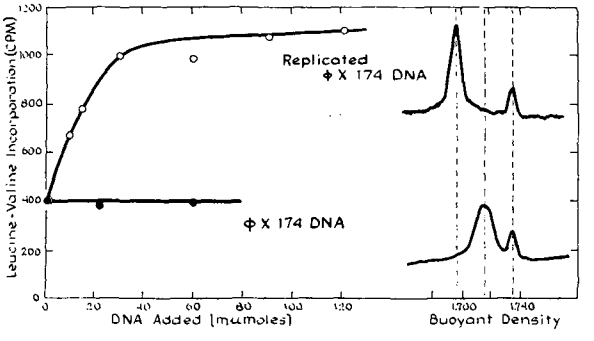

FIGURE 4. Activation of ϕX DNA by replication with DNA polymerase. ϕX DNA was replicated 3-fold and isolated as described under Fig. 3. Assays of DNA samples for activity were as in Fig. 2. Buoyant densities, determined as in Fig. 2, were: ϕX DNA, 1.715 g cm^{-3}; replicated ϕX DNA, 1.697 g cm^{-3}.

acid incorporation in the coupled SPR system under conditions where the original ϕX DNA is completely inactive.

INSUFFICIENT SYNTHESIS OF FREE RNA

Denatured T2 and ϕX DNA preparations direct RNA synthesis at a considerably lower rate than native T2 DNA (Table 2). Moreover, whereas double-stranded DNA is copied conservatively with the production of free RNA (Geiduschek, Nakamoto and Weiss, 1961; Chamberlin and Berg, 1963), the initial product of single-stranded DNA transcription is a hydrogen-bonded "hybrid" complex of RNA and DNA (Warner et al., 1963; Chamberlin and Berg, 1963), so that little or no free RNA is produced until the RNA to DNA ratio exceeds 1. Although no direct evidence is yet available, it seems likely that RNA complexed in such a hybrid would be inactive in stimulating amino acid incorporation. The observed inactivity of denatured and single-stranded DNA in the coupled SPR system

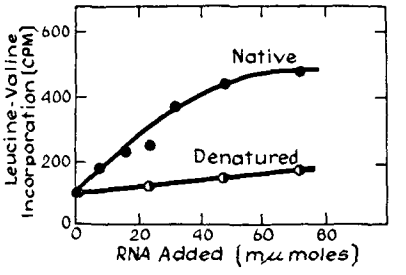

FIGURE 5. Stimulation of amino acid incorporation in the SPR system by isolated RNA directed by native and heat-denatured T2 DNA. RNA was synthesized in the presence of native and denatured T2 DNA, isolated, and assayed as in Fig. 1. The final preparations contained DNA at levels of 20% (native template) and 15% (denatured template) of the total nucleotide content.

might therefore (as suggested by Warner et al., 1963) be merely the result of insufficient free RNA production rather than synthesis of inactive RNA.

This explanation, however, is not supported by experiments in which enzymatically synthesized RNA was assayed directly in the SPR system in the absence of RNA polymerase and DNA. Isolated RNA preparations synthesized under the direction of native and denatured T2 DNA differ markedly in their ability to stimulate amino acid incorporation (Fig. 5). Comparison of the slopes of the two curves indicates that RNA directed by native template is approximately seven times more active than RNA directed by denatured DNA.

In the experiment summarized in Table 4, the activity of the free RNA produced in a two-fold synthesis with ϕX DNA was compared to the activity of RNA directed by native T2 DNA. Again, the T2 RNA is considerably more active, on

TABLE 4. ACTIVITIES OF ϕX 174 AND T2 RNA IN THE SPR SYSTEM

DNA added		RNA	"Free"	Isoleucine	RNA
Source	Amount	synthesized	RNA	*incorporated*	*specific activity*
	mμmoles	mμmoles	mμmoles	μμmoles	Δ ileu incorporation / "free" RNA added
None	0	—	—	2	—
T2 phage	6	19	19	20	0.9
ϕX 174	15	29	14	4	0.1*

RNA synthesis in the presence of RNA polymerase and the DNA template indicated in the first column was allowed to proceed until the level of total RNA (measured by incorporation of C^{14}-AMP) was as shown in the second column. The entire reaction mixture was then added to the pre-incubated SPR system described below, and amino acid incorporation was measured. Estimation of "free" RNA was based on the assumption (see text) that in the presence of double-stranded DNA all of the RNA produced is free, whereas in the presence of single-stranded DNA, one DNA equivalent of the total RNA remains bound in an RNA-DNA complex. This assumption leads to a minimum "free" RNA value and therefore a maximum specific activity for the RNA directed by single-stranded DNA. The SPR system was as in Table 1, except that C^{14}-L-isoleucine (1.4×10^7 cpm/μmole) was the only radioactive amino acid. The background level of amino acid incorporation was reduced by pre-incubating the system for 10 min at 37° before adding C^{14}-isoleucine and the RNA to be assayed (Ning and Stevens, 1962).

* A control experiment demonstrated that the presence of ϕX DNA:RNA hybrid did not inhibit the stimulation of amino acid incorporation by active T2 RNA, suggesting that the apparent inactivity of "free" ϕX RNA is not due merely to inhibition by the hybrid. The same argument applies to the results shown in Table 6.

a nucleotide basis, than the ϕX RNA. These findings demonstrate that the low activity of denatured and single-stranded DNA in the coupled SPR system is not due to insufficient RNA synthesis, but rather results from the low activity of the RNA which these DNA preparations direct.

ERRORS IN TRANSCRIPTION OF SINGLE-STRANDED DNA

As a possible explanation for the above findings, it was considered that the RNA polymerase might be unable to accurately copy a single-stranded DNA template, the result being an inactive RNA product containing non-specific deletions, substitutions, or additions in its nucleotide sequence. This possibility was suggested by the observation that single-stranded, but not double-stranded DNA supports the synthesis of polyadenylate in the presence of RNA polymerase and ATP as the only triphosphate (Chamberlin and Berg, 1962). When all four triphosphates are present, the RNA directed by single-stranded DNA appears to have the predicted nucleotide composition; however, small deviations from complementarity might not be detectable by this measurement. As a more sensitive test for accurate transcription, RNA preparations directed by native and denatured T2 DNA were analyzed for differences in the frequencies of eight of the sixteen possible dinucleotides (Table 5). The frequency of the ApA pair, which should be a sensitive indicator of polyadenylate synthesis, does not increase upon denaturation of the DNA template. In general, there appears to be no significant change in the frequency of any of the dinucleotides examined, and the values shown are in reasonable agreement with those reported previously for both T2 RNA (Weiss and Nakamoto, 1961) and T2 DNA (Josse, Kaiser and Kornberg, 1961).

Unfortunately, the extent to which dinucleotide frequency analysis can detect subtle changes in sequence is limited (Josse, Kaiser and Kornberg, 1961). With this reservation, there appear to be no gross differences in the accuracy with which native and denatured DNA are copied. In particular, the non-specific insertion of adenylate clusters during the transcription of denatured DNA can be excluded to within about 3%.

"NON-CODING" DNA.

The foregoing results, as well as the earlier experiments of Chamberlin and Berg (1962), suggest that single-stranded ϕX DNA is accurately copied by the polymerase. Its inability to direct active RNA synthesis might be explained, however, by the fact that in vivo, the DNA of the mature phage may be the "non-coding" strand in the synthesis of ϕX proteins. Following infection, the single-stranded phage DNA is converted to a "replicative form", presumably a double helix resulting from synthesis of the complementary DNA strand (Sinsheimer et al., 1962). Spiegelman and co-workers (this symposium) have reported that this newly synthesized strand alone is responsible for directing the subsequent synthesis of messenger RNA. The inactivity, in vitro, of the ϕX DNA could conceivably be associated with its passive role in the infected cell; the activity of the synthetic double-stranded ϕX DNA might then be ascribed not to its secondary

TABLE 5. DINUCLEOTIDE FREQUENCY ANALYSES OF RNA DIRECTED BY NATIVE AND DENATURED T2 DNA

Dinucleotide	Native	Denatured	Weiss and Nakamoto	Josse et al. (T2 DNA)
ApA	0.38	0.34	0.37	0.35
UpA	0.27	0.31	0.26	0.28
GpA	0.21	0.18	0.18	0.18
CpA	0.15	0.17	0.19	0.19
Sum	1.01	1.00	1.00	1.00
ApC	0.28	0.25	0.27	0.27
UpC	0.34	0.37	0.34	0.32
GpC	0.22	0.19	0.21	0.22
CpC	0.17	0.19	0.17	0.19
Sum	1.01	1.00	0.99	1.00

RNA synthesis in the presence of RNA polymerase and native or denatured T2 DNA was carried out under the conditions of the SPR system (see legend, Table 2) in separate reaction mixtures containing either ATP-α-P^{32} or CTP-α-P^{32} in addition to the other three unlabeled triphosphates. After addition of carrier protein, the reaction products were precipitated with trichloroacetic acid, washed, and incubated 18 hr at 37° in 0.3 N KOH. Following neutralization with perchloric acid and centrifugation to remove $KClO_4$, an aliquot of each hydrolysate was mixed with a solution containing the four 2′(3′) mononucleotides to serve as markers, and subjected to paper electrophoresis in pH 3.5 citrate buffer. Spots corresponding to the four mononucleotides were located with a UV lamp, cut out, and eluted with 0.01 N HCl for 4 hr, and an aliquot of each solution was plated and counted. Recovery of counts on electrophoresis was >98%, except in the hydrolysate from the reaction containing native DNA and ATP-α-P^{32}, from which the recovery was 87%.

structure, but rather to the presence of the complementary or "coding" strand.

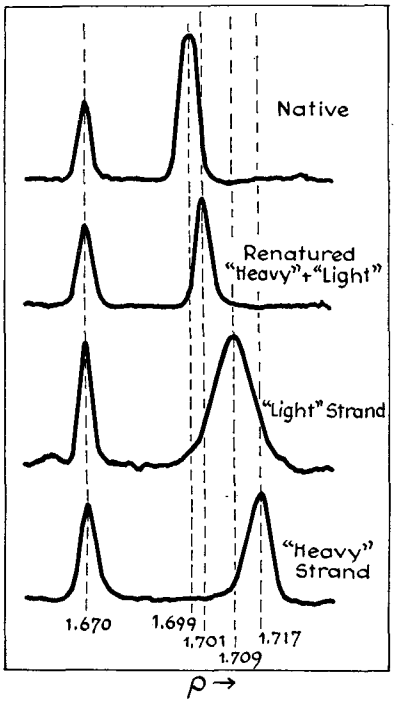

FIGURE 6. Buoyant densities of phage α DNA fractions in CsCl. Densities were determined as in Fig. 2, using dAT copolymer (ρ = 1.670 g cm^{-3}) as a reference. Renatured DNA was prepared by incubating equimolar quantities of the "light" and "heavy" fractions (DNA concentration 15 μg/ml) in saline-citrate 0.4 N in Na^+ at 45° for 2 hr, and then cooling the solution to room temperature over a second 2 hr period.

Evaluation of this possibility would require isolation of the "coding" strand to allow a comparison of its activity with that of the complementary DNA from the phage under identical conditions. Because of the technical difficulty of achieving strand separation in enzymatically synthesized DNA, such experiments were not feasible with φX. They could be carried out, however, using DNA from the *Bacillus* phage α. Cordes, Epstein and Marmur (1961; 1962) have shown that denaturation of this DNA leads to the appearance of two components, which differ in buoyant density and can be separated by chromatography on a methylated serum albumin column. The properties of the isolated fractions suggest that they represent the two complementary strands of the phage DNA. Dr. Marmur generously provided us with samples of native phage α DNA and the isolated "light" and "heavy" fractions for the following experiments.

Figure 6 illustrates the CsCl density gradient patterns obtained with native phage α DNA, the "light" and "heavy" fractions, and renatured DNA produced by incubating equal quantities of the two isolated fractions together at elevated temperature. The renatured material bands as a single component with a density close to that of the native DNA.

When assayed in the coupled SPR system, the native α DNA produces a stimulation of amino acid incorporation comparable to that observed with native T2 DNA (Fig. 7). Neither the "light" nor the "heavy" fraction alone shows activity. The renatured material, however, exhibits about two-thirds the activity of the native phage α DNA. The

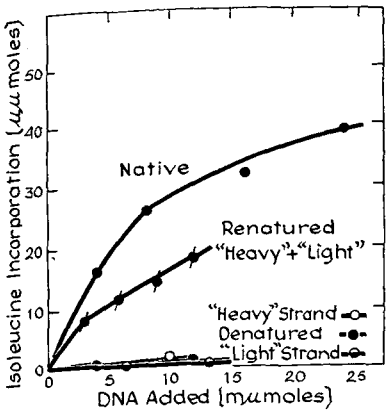

FIGURE 7. Reactivation of denatured phage α DNA by recombination of isolated single strands. The various α DNA fractions (see Fig. 6 and text) were assayed in the coupled SPR system, pre-incubated as in Table 4 prior to the addition of C^{14}-isoleucine, RNA polymerase and DNA.

inactivity of a heat-denatured sample of the native DNA shows that the presence of both strands in the denatured state is not sufficient to produce the activity seen upon renaturation.

Since there is no measure of RNA production in this experiment, the inactivity of the "light" and "heavy" fractions could again be attributed to hybrid formation or insufficient RNA synthesis. However, assays of the free RNA produced in the presence of the various α DNA fractions demonstrate that RNA directed by native or renatured material is four to five times as active in stimulating amino acid incorporation as the free RNA directed by either the "light" or "heavy" fraction alone (Table 6).

Therefore, although the "heavy" fraction probably represents the "coding" strand of the phage DNA (see Tocchini-Valentini and co-workers, discussion following Hall, this volume), neither the "light" nor the "heavy" fraction alone can serve in vitro as a template for active RNA; activity is manifested only when both are combined in a double-stranded structure. These results strongly support the conclusion that a single strand of DNA is incapable of directing the enzymatic synthesis of active RNA.

Changes in RNA Chain Length

With single-stranded DNA as a template, it is conceivable that the enzyme might stop and start more frequently or, alternatively, might be unable to release the template at customary stopping points, resulting in synthesis of RNA either smaller or larger than the product directed by native DNA. Other parameters being equal, the size of the RNA might be expected to influence significantly its activity in the SPR system. Studies on stimulation of amino acid incorporation in similar systems by synthetic polynucleotides have shown that most of the activity of a heterogeneous polynucleotide preparation can be attributed to the fraction which sediments most rapidly in a sucrose gradient, the slower-sedimenting fractions showing little or no activity (Matthaei et al., 1962; Jones and Nirenberg, 1962; Martin and Ames, 1962).

Zone centrifugation of RNA polymerase reaction products in sucrose gradients (Britten and Roberts, 1960) suggests that there may be a significant difference in the size distribution of RNA directed by native and denatured T2 DNA (Fig. 8). A rapidly sedimenting fraction, present in a substantial amount in the RNA directed by native DNA, is almost completely absent from the RNA directed by the denatured material.

It is not yet established that this size difference is responsible for the disparity in activity of the two preparations, nor is it clear why the more slowly sedimenting material should be less active. Preliminary experiments, however, suggest that the slower components are less readily bound to ribosomes than the rapidly sedimenting material. Since RNA must presumably be complexed to ribosomes in order to direct amino acid incorporation, this observation could explain the observed differences

TABLE 6. ACTIVITIES IN THE SPR SYSTEM OF RNA SYNTHESIZED WITH PHAGE α DNA FRACTIONS

DNA Added		RNA synthesized	"Free" RNA	Isoleucine incorporated	RNA specific activity
Fraction	Amount				Δ ileu incorporation / "free" RNA added
	mμmoles	mμmoles	mμmoles	μμmoles	
None	0	—	—	2	—
Native	9	29	29	31	1.0
"Light"	16	38	22	7	0.2
"Heavy"	12	25	13	5	0.2
Renatured	10	20	20	20	0.9

RNA synthesis, estimation of free RNA, and assay of RNA activity in the SPR system were carried out as in Table 4.

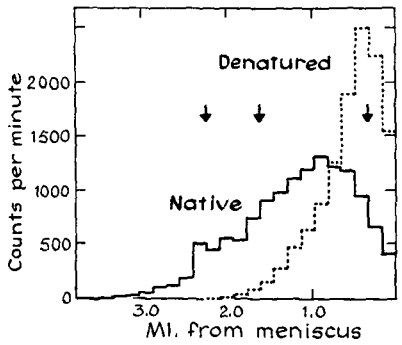

FIGURE 8. Zone sedimentation of RNA directed by native and denatured T2 DNA. RNA containing 8-C^{14}-AMP (3.0×10^6 cpm/μmole) was synthesized in the presence of native and denatured T2 DNA and isolated as in Fig. 1. Samples of the two preparations in volumes of 0.05 ml each were layered onto separate 4.8 ml linear 5–20% sucrose gradients, buffered at pH 7.8, and centrifuged 150 min at 39,000 rpm in the SW 39 swinging bucket rotor of a Spinco Model L refrigerated (5°) centrifuge. Fractions of 0.16 ml were collected through an orifice in the bottom of each tube, precipitated with ice-cold 3% perchloric acid, and collected and washed with cold water on Millipore filters for counting. The distributions of counts in the two gradients have been superimposed in the figure for comparison. Total counts shown in the figure are 13,470 (native template) and 12,650 (denatured template), representing >90% recovery of the applied RNA in each gradient. Vertical arrows indicate positions in the gradient approximately corresponding to (from right to left) sedimentation coefficients of 4S, 16S and 23S; these positions were estimated by sedimenting samples of purified ribosomal and amino acid-acceptor RNA from *E. coli* under conditions identical to those of the experiment.

in activity, although quantitative evaluation of the effect must await further study.

DISCUSSION

Although it is not yet clear why RNA directed by denatured DNA should be smaller than that directed by a double-stranded template, the following points may be pertinent to an explanation. Kinetic studies (Hurwitz et al., 1962; Wood and Berg, unpublished results) demonstrate that the RNA polymerase binds single-stranded DNA up to 50 times more strongly than double-stranded DNA. The enzyme, therefore, can probably begin copying a single-stranded DNA at any point on the chain. In copying a double-stranded template, the enzyme probably starts preferentially at single-stranded regions, and these should occur most frequently at the "frayed" ends of the helix. Since in these experiments the number of enzyme molecules is from about 2–20 times as great as the number of DNA molecules, simultaneous chain initiation at many points on the single-stranded template might result in an RNA population of shorter chain length. Because of the low rate of RNA synthesis with single-stranded DNA, it is technically difficult to avoid such an effect by working at low enzyme concentrations where the DNA template would be in excess. The unanswered questions on RNA size emphasize our almost complete lack of knowledge concerning the mechanisms of chain initiation and termination in DNA-directed RNA synthesis. It is to be hoped that further study of these phenomena will lead to a better understanding of how DNA transcription is controlled within the cell.

The preliminary observations on differences in RNA size do not exclude the interesting possibility, raised in the course of these experiments, that single-stranded DNA is inactive not only because of idiosyncrasies of the enzymatic copying mechanism in vitro, but because synthesis of active RNA requires information contributed by both strands of a DNA double helix. Experiments presented in this symposium have been interpreted to suggest that bacteriophage messenger RNA in vivo represents predominantly a complementary copy of only one of the two strands of the phage DNA. These experiments do not exclude the possibility, however, that this RNA, to be functional, must contain short sequences directed by the other DNA strand as well. The question of whether the RNA polymerase can copy intermittently from one DNA strand or the other in the course of synthesizing an RNA molecule can perhaps be answered by more detailed studies on the enzyme mechanism and the physical structure of the RNA product.

In closing, it should be emphasized that the relation of the amino acid incorporation reaction described in these studies to specific protein synthesis in the intact cell remains unclear. As direct evidence for the in vivo role of RNA polymerase, the fact that RNA made with the enzyme can stimulate amino acid incorporation is encouraging but not conclusive, particularly since it was shown that random copolymers of the four ribonucleotides can produce a similar stimulatory effect (Jones and Nirenberg, 1962). It is therefore crucial that de novo synthesis of specific proteins under the influence of DNA-directed RNA in the SPR system be demonstrated. Progress toward such a demonstration has been reported by several workers who have observed increases in the level of an inducible enzyme in bacterial extracts supplemented with RNA polymerase and DNA from induced cells (Eisenstadt, Kameyama and Novelli, 1962; Imai, Yura and Marushige, 1963); however, it is difficult to prove that such increases represent de novo enzyme synthesis. Since several well-characterized enzymes, absent from *E. coli* extracts, are specifically induced under the influence of the incoming DNA following T2 phage infection (Kornberg et al., 1959; Flaks, Lichtenstein and Cohen, 1959), the system used in these studies offers promise for further work in this direction.

SUMMARY

RNA synthesized with RNA polymerase under the direction of several native DNA preparations markedly stimulates amino acid incorporation in a soluble protein-ribosome system (SPR system). Little or no stimulation is observed with RNA directed by any denatured or single-stranded DNA. The ability to direct active RNA synthesis is restored upon conversion of this DNA to a double-stranded form by replication with DNA polymerase or by renaturation of the denatured material. Further support for a direct correlation between DNA activity and secondary structure comes from the finding that when the two complementary strands of an active DNA are isolated and tested separately, neither alone will direct active RNA synthesis, but the ability to do so can be restored by renaturing them to re-form a double-helical structure. The physical basis for differences in RNA activity is still unclear. RNA preparations directed by native and denatured T2 DNA do not differ significantly in their dinucleotide frequencies, but do differ in their size distributions; the RNA directed by the denatured DNA is appreciably smaller and more homogeneous in size than that directed by native DNA.

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